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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/510,655	11/30/2004	Hans Groenlund	24741-1536	9022

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EXAMINER	
ROONEY, NORA MAUREEN	
ART UNIT	PAPER NUMBER
1644	

SHORTENED STATUTORY PERIOD OF RESPONSE	MAIL DATE	DELIVERY MODE
31 DAYS	04/02/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

Office Action Summary	Application No.	Applicant(s)	
	10/510,655	GROENLUND ET AL.	
	Examiner	Art Unit	
	Nora M. Rooney	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *30 January 2006*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-6 and 9-14* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *1-6 and 9-14* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

1. Applicant's amendment filed on 10/08/2004 is acknowledged.

2. Claims 1-6 and 9-14 are pending.

3. Restriction is required under 35 U.S.C. 121 and 372.

4. This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

5. In accordance with 37 CFR 1.499, applicant is required, in response to this action, to elect a single invention to which the claims must be restricted.

Group I, Claims 1-6 and 9-10, drawn to a microparticle comprising a bead consisting essentially of a three dimensionally cross-linked carbohydrate and an allergen which is covalently bound to the bead, wherein the allergen is derived from plant pollen and a medicament.

Group II, Claims 11-12, drawn to a diagnostic test system comprising administering the microparticles comprising a bead consisting essentially of a three dimensionally cross-linked carbohydrate and an allergen which is covalently bound to the bead, wherein the allergen is derived from plant pollen and measuring the cell mediators released in response thereto.

Group III, Claims13-14, drawn to a method of vaccinating a subject comprising administerng an effective amount of the microparticle comprising a bead consisting essentially of a three dimensionally cross-linked carbohydrate and an allergen which is covalently bound to the bead, wherein the allergen is derived from plant pollen and a medicament.

6. The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The invention of Group I was found to have no special technical feature that defined the contribution over the prior art of King et al. (PTO-892, Reference U) and Nordvall et al. (PTO-892, Reference V).

King et al. teaches a microparticle comprising a bead consisting essentially of a three dimensionally cross-linked carbohydrate (Sepharose bead) and an allergen (Dactylis glomerata protein) which is covalently count to the bead, wherein the allergen is derived from plant pollen (grass pollen) (In particular, abstract).

Nordvall et al. teaches a microparticle comprising a bead consisting essentially of a three dimensionally cross-linked carbohydrate (Sepharose bead) and an allergen (Timothy Grass pollen allergen) which is covalently count to the bead, wherein the allergen is derived from plant pollen (Timothy grass pollen) (In particular, abstract).

Since Applicant's inventions do not contribute a special technical feature when viewed over the prior art they do not have a single general inventive concept and so lack unity of invention.

7. Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Nora M. Rooney whose telephone number is (571) 272-9937. The examiner can normally be reached Monday through Friday from 8:30 am to 5:00 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on (571)

272-0841. The fax number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

March 25, 2007

Nora M. Rooney, M.S., J.D.
Patent Examiner
Technology Center 1600

Maher M. Haddad
MAHER M. HADDAD
PRIMARY EXAMINER

Notice of References Cited	Application/Control No. 10/510,655	Applicant(s)/Patent Under Reexamination GROENLUND ET AL.	
	Examiner Nora M. Rooney	Art Unit 1644	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	King et al. 'Demonstration of reaginic antibodies on human basophils by immune adherence to allergen-coated Sepharose beads.' Clin. Allergy 6(4):339-348, 1976.
	V	Nordvall et al. 'Timothy-specific IgG antibody levels vary with the pollen seasons.' Allergy 41(8):575-580, 1986.
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Clinical Allergy, 1976, Volume 6, pages 339–348

Demonstration of reaginic antibodies on human basophils by immune adherence to allergen-coated Sepharose beads

M. KING,* N. ALAYE† *and* R. AUGUSTIN

Sub-Department of Immunology, Liverpool University

Summary

Human basophils purified by gradient density centrifugation and differential glass bead adherence were interacted with grass pollen allergen in particulate form prepared by covalent coupling of *Dactylis glomerata* protein concentrate to Sepharose beads.

Basophils from allergic subjects were found to interact specifically with the allergen-coated bead surface in a manner so highly characteristic that false negatives with basophils from non-allergic subjects or subjects with unrelated allergies were not encountered. Microscopic examination indicated that the specifically stained malleable basophils had adapted in a multi-point attachment to the rigid bead surface by becoming one-sidedly flattened against it. When using basophils from highly pollen sensitive subjects all the beads carried basophils and sometimes as many as thirty per bead. The percentage of beads with basophils and the number of basophils per bead roughly correlated with clinical history and skin tests. Immunocytoadherence of the basophils to the allergen-coated beads was specifically inhibited by anti-IgE and anti-allergen antibodies, including reagins.

Introduction

More tests are now available for measuring serum reagins than for probably any other type of antibody (Augustin, 1967, 1973). However, clinical reactions are not caused by free serum reagins, but by the release of vasoactive amines and other highly active substances during the interaction of allergens with reagins fixed to blood basophils and tissue mast cells. Allergen-mediated histamine release from sensitized chopped human lung (Augustin, 1967, 1973) and blood leucocytes (Lichtenstein & Osler, 1964) are well-established methods for demonstrating allergic hypersensitivity and it has been shown conclusively that of all the blood cells only basophils have receptors for IgE (Pruzansky & Patterson, 1970; Ishizaka *et al.*, 1972). A number of experimental findings have led to the assumption that allergens (and other agents) capable of initiating histamine release from basophils must be capable of bridging two or more of the IgE molecules on the cell surface (Ishizaka & Ishizaka, 1968; Stan-

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worth, 1973). We describe here the development of a test system which confirms this by direct visualization.

Materials and methods

Human blood

This was obtained by venepuncture from normal and allergic volunteers known to be clinically sensitive to grass pollens and giving positive prick tests to extracts of *Dactylis glomerata*. Medication with antihistamines and/or cromoglycate did not seem to interfere with our test, nor did it seem to matter whether the tests were done during or outside the hay fever season.

Reagents

Ficoll was obtained from Sigma Ltd and was prepared freshly each week as a 9% (w/v) solution in deionized water.

Triosil was obtained in 20 ml vials from Nyegaard & Co. A.S., Oslo. The contents of a vial were mixed with 24 ml of deionized water and the solution used the same week.

CNBr-activated Sepharose beads were purchased from Pharmacia, Uppsala, Sweden, *glassbeads* of about 0·7 mm diameter (Ballotini No. 9) from Jencons (Scientific) Ltd, Hemel Hempstead, Herts, and *methylcellulose* of viscosity 15 cP USP grade from Dow Chemicals, Cheshire.

Solutions

Tris A buffer was prepared according to Lichtenstein & Osler (1964). It contains 3·75 g Trisma 7·6, 6·95 g NaCl, 0·37 g KCl and 3 g human serum albumin (HSa) in 1 l of deionized water, adjusted to pH 7·4 with N HCl.

Buffered Ca^{++} and Mg^{++} free EDTA (referred to as R-buffer) was prepared according to Rabinowitz (1964). It contains 0·2 g disodium EDTA, 8·0 g NaCl, 0·2 g KCl, 1·15 g Na_2HPO_4 and 0·2 g KH_2PO_4 in 1 l of deionized water (pH = 7·4).

Apparatus

Glassware. All glassware was carefully washed and siliconed as described by Rabinowitz (1964), including the glass beads, using water soluble 'Siliclad' (Clay-Adams). Used beads from the columns described below were first thoroughly rinsed in saline to remove residual cells and proteins, then boiled with dilute 'Teepol' detergent before finally rinsing and washing as described above (Shortman *et al.*, 1971).

Containers. Short round- or flat-bottomed polycarbonate centrifuge tubes, 10–12 ml and 1–2 cm diameter, were used as well as 25 ml plastic screw top 'Universal' bottles, all from Sterilin Products, Richmond, Surrey, from whom we also purchased the disposable Pasteur pipettes.

Methods

Grass pollen allergen concentrate. This was prepared essentially as described by Augustin & Hayward (1962) and Augustin (1973). As described there and detailed earlier (Augustin, 1959; Augustin & Hayward, 1962), large amounts of impurities together with up to 10% activity were removed by a preliminary 1 min extraction with water. The subsequent combined extracts were precipitated by 90% saturation with

ammonium sulphate. After desalting and freeze-drying, this preparation may be stored for prolonged periods at 4°C and as much as $1{\cdot}5–2{\cdot}0 \times 10^6$ Noon units (Noon, 1911) are recovered per 1 g of pollen.

Particularized allergen (Axen, Porath & Ernbach, 1967). A 0·4 ml aliquot of the desalted pollen protein concentrate freshly prepared by the method described above (freeze-drying omitted) and containing by skin test 10^7 Noon units/ml (Noon, 1911) was dialysed for 24 hr (magnetic stirrer) against 0·1 M $NaHCO_3$/0·5 M NaCl, pH 8·0 (two changes) and then diluted to 5 ml with the same buffer. This was added to 1 g of washed swelled CNBr-activated Sepharose 4B beads in a 10 ml screw top test tube. After rotating at 4°C for 16 hr the suspension was filtered and washed with bicarbonate/NaCl buffer. The suspension was then incubated for 2 hr (rotation) at room temperature with 50 ml of 1 M ethanolamine, pH 8·0 and followed by five cycles of washing with 0·1 M acetate/1 M NaCl pH 4·0 alternating with 0·1 M borate/1 M NaCl pH 8·0, until the optical density 280 readings of the filtrates were negligible. The beads were finally suspended in 0·1 M borate/1 M NaCl pH 8·0 for storing at 4°C, NaN_3 being added at a concentration of 0·02% as a preservative. When required for the basophil immune adherence test (BIAT), a suitable aliquot of the suspension was washed four times in R-buffer to remove NaN_3. Human serum albumin was similarly bound to Sepharose beads using 10 mg/g of dry gel.

Inactivated uncoupled beads were prepared by reacting with ethanolamine at pH 8·0 for several hours (end on end rotation) followed by several wash cycles alternating between pH 4·0 and 8·0.

Our conjugated beads have been stored in the cold room (4°C) in the presence of azide for over a year without deterioration.

Basophil enrichment. Fifty millilitres of blood drawn into a 50 ml plastic syringe containing 500 units of heparin (no preservative) and 5 ml of 1% methylcellulose in Hanks' balanced salt solution (HBSS) was gently mixed and allowed to sediment in an inverted position for 30–35 min at room temperature. The upper plasma layer containing the bulk of the leucocytes was then gently pushed out through a soft piece of narrow tubing into polypropylene tubes (May, Lyman & Alberto, 1970). After centrifugation for 10 min at 150 *g* (4°C), the combined cell sediments were resuspended in 4 ml of the supernatant plasma previously clarified by centrifugation at 1000 *g*. Of the leucocytes, 60–75% were recovered and the bulk of the polymorphonuclear cells were then removed by differential centrifugation according to Boyum (1968). This step gave basophil/leucocyte ratios of about 1·4–3·5 in 60–70% yield and the final fractionation was performed on glass bead column, essentially according to a method originally devised by Rabinowitz (1964) for the fractionation of lymphocytes. At a temperature of 37°C the cell plasma suspension was layered on to the glass bead column immediately after washing it with Tris-A buffer. After penetration, 30 min was allowed for equilibration and active adherence. For the first elution 30 ml of 20% of the reserved cell-free plasma in Tris-A buffer was used in place of Rabinowitz's HBSS, at a speed of 15 drops/min. The resulting first eluate contained non-adherent lymphocytes and the bulk of residual platelets, but no monocytes or basophils. This was followed by elution with 20 ml of Na^{2+}- and Mg^{2+}-free EDTA solution (R-buffer) at a speed of 25 drops/min and yielded the bulk of the basophils in the second eluate together with the more adherent lymphocytes and some monocytes, the monocytes and damaged cells emerging last. From 50 ml of blood $1{\cdot}5–10 \times 10^5$ basophils were recovered, representing overall yields of 12–30%. Of the suspended cells 8–25% were basophils with

viabilities above 95% by dye exclusion. After washing four times in R-buffer the cells were suspended in 1 ml of the same buffer.

Basophil staining. Neutral red staining was performed according to Shelley (1965), using 6 mg of neutral red dissolved in 50 ml of 1% (w/v) phosphate buffer, pH = 5·4.

Formaldehyde fixative. A 0·5 ml aliquot of 40% (v/v) CH_2O were mixed with 3·5 ml of 1% phosphate buffer, pH 5·4.

Neutral red/saponin solution. One millilitre of the above neutral red solution was added to 0·025 ml of saponin solution prepared by dissolving 40 mg of saponin in 1 ml of the 1% pH 5·4 phosphate buffer.

Acridine orange (AO) staining. Two drops of the cell suspension (> 10 000 cells/ml) were mixed with two drops of a 0·02% solution of AO in PBS, pH 7·2 and viewed under a fluorescent microscope (Fitzpatrick *et al.*, 1967; West & Augustin, 1970). Basophils can be distinguished very clearly from other polymorphonuclear cells, lymphocytes, monocytes and macrophages.

Skin testing was done by the prick method, using dilutions of a pollen standard containing 100 000 Noon units per ml (Augustin, 1967, 1973).

Tests for viability were done by dye exclusion according to Schreck (1936).

Cell counts. Total leucocytes were counted in a Neubauer counting chamber in the usual manner, and basophils in a Fuchs–Rosenthal chamber after staining according to Shelley (1965). In the absence of RBC, 0·05 ml of the cell suspension ($> 10^5$ basophils/ml) were mixed with 0·4 ml of neutral red solution while in the presence of RBC the saponin/neutral red solution was used in place of neutral red alone.

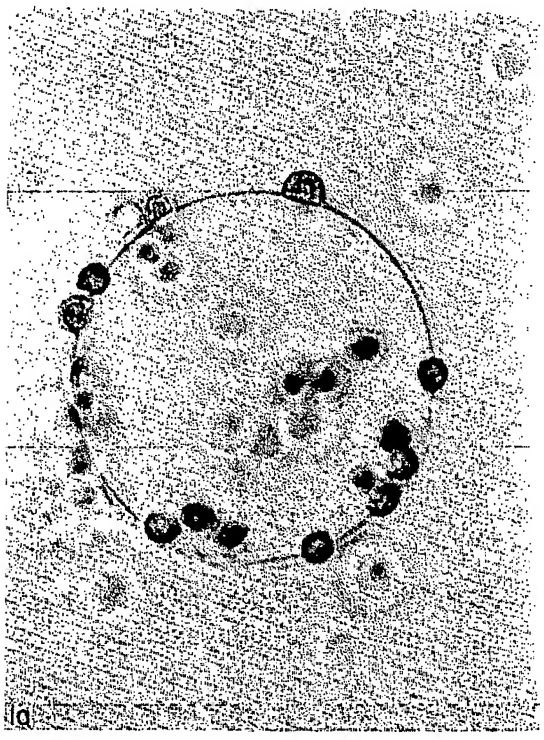

FIG. 1a

FIG 1b

Fig. 1. Specific immune adherence of basophils from a strongly grass pollen sensitive subject to Sepharose 4B beads irreversibly coated with the relevant pollen allergens (*Dactylis glomerata*). (a) × 300. By varying the plane of focus twenty-five basophils could be seen to be characteristically attached to the upper surface of one single bead. (b) × 500. Close-up of a bead with four attached basophils to show more clearly the very characteristic manner of basophil attachment.

Results

Immune adherence test procedure

If at all possible the immune adherence test was done immediately the basophils had been purified although it has been possible to keep the cells in good shape in the EDTA buffer overnight at 4°C and use them then. However, viability may then drop and bead adherence was never quite as high as when the basophils were used right away. One millilitre of the washed cell suspension containing a minimum of 10^5 basophils in R-buffer were incubated with a volume of washed allergen-coated beads in R-buffer giving a ratio of beads to basophils of 1:30. Usually the selected number of the appropriate washed beads were sedimented in 10 ml siliconed short, wide round- (or flat-) bottomed tubes, overlayered with the basophil suspension and then very gently mixed by carefully rotating and rolling the tube by hand. This was followed by a 5 min incubation at 37°C and the gentle mixing repeated. The tubes were then spun for 4

min at 150 *g* (bucket swing-out type of head) and this was followed without further disturbance by a 40 min incubation at 4°C. The supernatants were then drawn off with a Pasteur pipette and beads and cells very gently suspended in the remaining last drop of supernatant by gently rocking to and fro. The cells were then stained with neutral red (or AO) by gently mixing them with 0·4 ml of the neutral red (and 0·05 ml of the fixative solution). Basophil-bead adherence may then be determined quantitatively in a Fuchs–Rosenthal chamber. Under the given conditions basophils could be quite clearly distinguished from all the other cells since quite apart from their characteristic grape-like appearance no other cells but basophils stain pink with neutral red. After staining with AO the orange fluorescing granules cover more or less completely all other structures and these orange fluorescing grape-like balls are again very different from the other mononuclear cells which show distinctive green fluorescing nuclei. A minimum of fifty beads were each time evaluated by scanning the whole field at a × 100 magnification, followed by checking at higher magnifications whenever desirable. Figure 1 shows the highly characteristic manner in which the basophils firmly cling in an obviously multivalent attachment to the bead surface. This can be already appreciated under the × 100 magnification, but is seen much more clearly on changing to a higher magnification. At × 400, and still better under oil (× 1000), it can be seen that the basophils actually become deformed and flattened against the bead surface in their multivalent attempt to accommodate to the rigid bead surface. The basophil membrane appears to become broken to some extent in the region of attachment, with some spilling of granules which however appear also to remain attached to the bead surface. It is therefore very easy to distinguish between true basophil-bead interaction and the accidental presence of non-reacted basophils and other cells near or on the bead

Table 1. Correlation of basophil adherence with skin tests and clinical history. Severity of clinical sensitivity to grass pollen indicated by number of plus signs, ? indicates probable lack of clinical sensitivity to pollen. Note that skin sensitivity is the higher the lower are the Noon units (Noon, 1911)

Patient	Clinical sensitivity to pollen	Skin tests: lowest pollen concentration giving +ve reaction (Noon units/ml)	Allergies other than to pollen	Drugs taken	% Beads with basophils
TOD	+++++	1	No	Antihistamines, cromoglycate	100
BROO	++++	10	Yes	Antihistamines, ACTH	100
RO	+++	100	No	Antihistamines	80
BERN	+++	10	No	Antihistamines	50
RES	+++	1000	No	Antihistamines	37
WES	++	100	Yes	Antihistamines	15
PHIL	?	1000	Yes	Antihistamines	3
CART	?	100	Yes	Antihistamines ACTH	0
JON	?	10 000	Yes	Antihistamines, ACTH, cromoglycate	0

surface. Non-reacted cells do not conform to the bead surface and remain spherical.

In a positive test where the basophils have been derived from a donor with reaginic antibodies for the allergen attached to the beads, 10–100% of the beads may have one to thirty basophils attached to them in this very characteristic manner. Table 1 shows that the percentage of beads with basophils attached to them and the number of basophils per bead increases with clinical sensitivity and was also roughly correlated with the severity of the skin test reaction to the allergen.

Specificity of the basophil immune adherence test

Three negative controls were used; in one the specific allergen-covered beads were replaced by unconjugated beads (inactivated with ethanolamine as described under Methods) and in the other by beads coupled to an unrelated antigen such as human serum albumin or haemocyanin: not in one single instance was a false positive obtained nor did we ever obtain a false positive result when reacting the basophils of non-allergic subjects with our pollen allergen-conjugated beads. In all three negative controls, all the beads were always completely free of cells, demonstrating clearly the specific nature of the reaction.

Furthermore, incubation of the basophils with anti-IgE serum (kindly supplied by the Drs Ishizaka) prior to reacting with the specific beads, inhibited the immune adherence, confirming the IgE specificity of the basophil-attached antibody. Similarly, allergen-coated beads failed to collect basophils on their surface when reacted previously with antisera for the allergens and this included some reaginic sera.

Discussion

The test described here may be regarded as a variant of the basophil rosette test described by Wilson *et al.* (1971, 1972) in which the allergen-coated RBC have been replaced by allergen-coated Sepharose beads.

Although rosette tests involving lymphocytes are by now well-established methods, basophil rosette tests pose special problems, firstly because usually only the very dilute and impure commercial allergen solutions used in skin testing are available and secondly because of the very low concentration of basophils in whole blood. The necessary intimate contact between the particularized reactants may therefore be prevented simply by crowding out with impurities. Tests are therefore subject to the availability of at least partially purified antigen concentrates and highly enriched basophil suspensions. A rapid method for the preparation of grass pollen allergen concentrates is indicated in the text. The iron carbonyl method used by Wilson *et al.* (1972) for basophil enrichment is only suitable for bloods with exceptionally high basophil counts and is replaced here by a three-step purification similar to the combination of methods used independently by Pruzansky & Patterson (1970) and Ishizaka *et al.* (1972), both groups applying the leucocytes to glass bead columns as a suspension in HBSS/plasma (containing Ca^{2+} and Mg^{2+}), a step considered necessary by Rabinowitz (1964) for the promotion of active adherence to the glass beads. We found, however, that this 30-min incubation and the subsequent elution of non-adherent cells with HBSS/plasma was detrimental to the basophils. By replacing HBSS in the 20% plasma mixture with the EDTA and glucose containing R-buffer no interference with basophil attachment resulted and this yielded a 10–30% pure basophil suspension in 15–25% overall yield and in excellent morphological condition. The viability of these cells by dye exclusion was always in the region of 94–98%. Preservation of histamine content was not tested

for, but surface IgE antibodies appear to have remained intact. At any rate, the basophils remained capable of specific immune adherence to the corresponding allergen-covered beads and failed to stick non-specifically to beads, even when stored overnight (4°C). Under appropriate conditions, the human basophil may thus prove more stable than hitherto expected although preservation of histamine content may prove to be a more stringent test for this than the retention of reagins, for instance Ishizaka & Ishizaka (1974) have shown that IgE molecules can be dissociated from their receptors at pH 4·0 without greatly damaging these receptors, although histamine release had been triggered off in the process.

Several advantages accrued from our replacing the RBC indicator cells with inert beads. Sepharose beads are stable and available commercially in an already activated form which makes coating with protein antigen a very easy and quick operation while antigen coating of mammalian cells has required great ingenuity (Coombs & Franks, 1969) and can easily result in incipient damage with subsequent non-specific stickiness and non-specific adherence. Moreover accidental juxtaposition of cells can sometimes mimic specific adherence and is not possible in the BIAT described here which is a kind of inverted rosette test in which the basophils surround the much larger antigen-coated beads. This led to a very characteristic mode of attachment which cannot be confused with chance non-specific adherence or juxtaposition. We found that the obviously multiple attachment of the malleable basophils to the rigid bead surface caused a characteristic deformation of the basophils and visually supports the previously postulated multivalent triggering of basophils (Ishizaka & Ishizaka, 1968; Stanworth, 1973). False positives were never encountered. On the other hand, the relatively large size is probably also the reason why the test is not as sensitive as one would wish and using beads of a size similar or even smaller than the basophils may be expected to improve sensitivity. Subjects with positive prick tests to not less than 1000 Noon units/ml reacted poorly in the present BIAT.

Contact with the appropriate allergen may cause not only histamine release from the basophils (or mast cells), but may also lead to degranulation and it has been reported that fewer than usual blood basophils are found during the hay fever season (Wilson *et al.*, 1971). We, on the other hand, have not become aware of such a decrease although we have isolated basophils from four of our volunteers on three and more occasions, some inside and some outside the hay fever season. One reason for this may be that most of our more sensitive volunteers appear to have been under corticoid and/or cromoglycate treatment when tested during the hay fever season. Although this must have affected allergen-mediated histamine release, it in no way interfered with our test which depends solely on maintaining surface IgE attachment. Even if histamine had been released from basophils collected during the hay fever season, this would not have interfered with our test although actual degranulation would have done so. Moreover, under our conditions, basophil membranes were well maintained, possibly stabilized by the drugs taken by the patients.

Serum IgE contents can vary between <100 and over 10 000 ng/ml. This is equivalent to about 4×10^{14} to 4×10^{16} molecules of free IgE/ml. And as there are usually between 5000 and 10 000 basophils/mm^3 of blood and about 20 000 IgE receptors/basophil (Ishizaka *et al.*, 1973), there can only be between $5\times10^6\times2\times10^4$ ($=10^{11}$) and 2×10^{11} basophil-bound IgE molecules/ml blood, i.e. there are probably 1000–100 000 as many free IgE molecules in the blood as are bound to the basophils. Of course, only some of these are antibodies and it may even be that the qualitative

antibody composition in serum differs from that on the basophils. Yet antigen-mediated histamine release requires the participation of cell-bound reagins (i.e. either blood basophils or tissue mast cells). From this it follows clearly that, once no more free IgE receptors are available (on basophils or mast cells), free reagins will protect just as well against allergen-mediated histamine release as do IgG and other non-IgE classes of antibodies of the relevant allergen specificity—although only the latter are usually referred to as 'blocking' antibodies. Yet the reaction of allergen entering the blood stream with basophil-fixed antibodies will be competed with—and therefore 'blocked' —by any allergen-specific free antibodies, including free serum reagins. Excess reagins produced during hyposensitization treatment in addition to non-IgE antibodies may therefore be not always as deleterious to the patient as is generally thought.

Acknowledgments

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Timothy-Specific IgG Antibody Levels Vary with the Pollen Seasons

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Serum samples were collected from eight grass pollen hypersensitive children during a 4-year period. The sera were assayed for contents of timothy-specific IgE antibodies by RAST. Timothy-specific IgG and IgA antibodies were quantified by a refined ELISA in which covalent binding of the antigen to the polystyrene solid phase had been performed. IgG antibodies were also assayed by a Sepharose-protein-A technique with radiolabelled timothy allergens as the antigen. It was possible to register clearcut seasonal variations with postseasonally boosted antibody levels not only of timothy-specific IgE but also of IgG antibody. Both IgG1 and IgG4 antibodies specific for timothy showed seasonal variations of a similar degree. It was not possible to register seasonal variations of the same magnitude of timothy-specific IgA antibodies.

Key words: ELISA; IgG-antibodies; surface-grafting; timothy.

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Pollens from wind-pollinating herbs appear seasonally in the atmosphere. In the Scandinavian countries the major causes of pollenosis are pollens from the deciduous trees, which are predominant in the spring time (March–June), and grass pollens, which appear in the summer (June–August). The intermittent yearly exposure to pollens causes the characteristic symptoms of hay fever, which are known to be well correlated to the pollen load (7). The seasonal allergen boost is also known to cause an elevation of the allergen-specific serum IgE level which after some time subsides. Thus seasonal variations in the pollen-specific IgE levels are caused (2).

With sensitive techniques it has been possible to show that not only allergen-specific IgE antibodies are raised in sera of atopics but also allergen-specific IgG and IgA antibodies (15). Due to the high levels of serum IgG, the accurate determination, especially of low IgG antibody concentrations, involves considerable problems caused by non-specific background binding. Thus, with many techniques, allergen-specific IgG antibody levels in sera of non-hyposensitized patients are registered within or only barely above background. Nevertheless, allergen-specific IgG-antibody concentrations may very well be just as high as the corresponding IgE antibody concentrations or even higher (15).

In the present work we have applied two highly sensitive assay techniques for detection of low concentrations of timothy-specific IgG antibodies: an enzyme-linked immuno-sorbent assay (ELISA) with recent modifications for improvement of sensitivity (10) and a Sepharose protein-A method (6). Sera from 4 consecutive years drawn from eight children with grass pollen hay fever were analysed for contents of timothy-specific IgG, IgA and IgE antibody and the results were related to the occurrence of pollen season.

MATERIAL AND METHODS

Patients

Eight children aged 7–16 years at the start of the study and judged to be grass pollen allergics by a typical history, positive RAST tests, positive skin prick, and conjunctival provocation tests, were included in the study. Blood was drawn annually on several occasions, before and after the grass pollen season during a 4-year period. The sera were kept frozen −18°C before analyses. During the 4 years the patients were given ordinary treatment with antihistamines and disodiumcromoglycate, none were treated with corticosteroids.

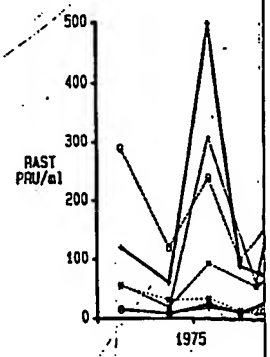
Antibody analyses

Timothy-specific serum IgE antibodies were assayed by RAST, (8, 18) using the commercially available Phadebas RAST kits (Pharmacia Diagnostics AB, Uppsala, Sweden). Allergen-coated paper discs were incubated with undiluted patient serum for 3 h, washed 3 times (in approximately 2.5 ml of saline containing 0.1% Tween 20), aspirated dry and after that incubated with 100 µl of ^{125}I-labelled Dε2-specific rabbit anti-IgE (1) (50,000–70,000 cpm/test tube) overnight. After washes (see above) the count rates were determined in a gamma scintillation counter. The counts were related to those of a reference serum of the Phadebas RAST reference system and expressed in PRU/ml.

ELISA was principally run as previously described by others (5) but with several important modifications for the improvement of the sensitivity of the assay. The antigen used was a refined preparation of timothy pollen (Spectralgen) kindly provided by Pharmacia Diagnostics AB, Uppsala, Sweden. The antigen was covalently bound to polystyrene microtitre plates according to a recent method of Larsson et al. (10). The method was based on surface grafting of reactive monomers to a polymeric substrate using polystyrene microtitre plates (Nunc Immunoplate II, Nunc, Denmark). The free radical mediated reaction was initiated and sustained by a 3 Mrad delivery, at 0.25 Mrad/h, from a ^{60}Co gamma-source. All monomeric solutions were kept free of oxygen throughout the grafting reaction.

Two kinds of surface-modified microtitre plates were used for covalent solid phase binding. The ELISA assays for total timothy-specific IgA and IgA antibody concentrations were run on acrylamide (Bio-Rad, Richmond, USA) grafted plates, which were activated with 1% aqueous glutaraldehyde and buffered to pH 7.6 at 37°C overnight. The plates were then extensively washed with distilled water in four 5-min cycles. The antigen was dissolved in a 0.1 M sodium phosphate buffer, pH 7.6, 100 µl of which was added to each well at the concentration 20 µl/ml. The binding step was allowed to continue overnight at +4°C. Inactivation of remaining aminogroup reactive sites was performed by addition of 100 µl 1 M ethanolamine, pH 8 to each well. Elimination of noncovalently bound material was done by three alternate washings with a 0.1 M sodium acetate buffer, pH 4 and a 0.1 M borate buffer, pH 8.

The ELISA assays for timothy-specific IgG1 and IgG4 were performed on crotonic acid (purum, Fluka AG, Busch, Switzerland) grafted microtitre plates. The plates were activated with EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl, Bio-Rad, Richmond, USA). The carbodiimide was added in the volume of 100 µl/well at a concentration of 10 mg/ml in 3 mM potassium phosphate buffer, pH 6.3. The plates were then incubated for 60 min at ambient temperature. After three washings with ice-cold redistilled water, 100 µl/well of the antigen was added at a concentration of 20 µl/ml in the pH 6.3 buffer. The binding reaction was allowed to continue overnight at 4°C. Inactivation and washing cycles were performed as for the acrylamide-grafted plates. In the assay serum was diluted 1/100 in an assay buffer solution (50 mM phosphate buffer pH 7.4, 0.15 M NaCl, 0.2% BSA, 0.1% Tween 20 and 0.02% NaN3). 100 µl of this dilution was incubated in each well for 3 h at room temp. after which three washings were performed (see above). The aforedescribed assay buffer solu-



tion was used also for th
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The antisera used,
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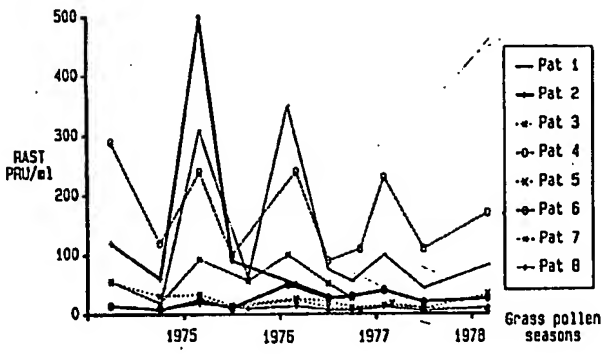

Fig. 1. Timothy-specific serum IgE antibody concentrations as measured by ELISA technique in eight atopic children during a 4-year period in relation to the pollen seasons.

tion was used also for the below described dilutions of sera and reagents. Bound IgG and IgA were detected with affinity purified anti-IgG and -IgA antisera, respectively, both at the concentration of 200 ng/ml. 100 µl was added to each well and the incubations were performed at 37°C for 1 h. In a second step an SPDP-coupled conjugate of alkaline phosphatase and F(ab)' fragments of affinity purified sheep anti-rabbit gammaglobulin (MIAB, Uppsala, Sweden) was used, 100 µl at the dilution 1/200.

IgG subclasses were detected using mouse monoclonals to the different IgG subclasses in the form of ascitic fluid (Seward Laboratory, London, England). Preliminary experiments indicated significant presence of only IgG1 and IgG4 antibodies, which was also in agreement with findings of others (4). Thus interest was focused on these two subclasses. 100 µl serum diluted 1/10 was incubated overnight and after washings 100 µl of the monoclonals was added, each at the dilution 1/2000. In the next step 100 µl of a rabbit anti-mouse antiserum diluted 1/2000 was used, the binding of which was traced using the sheep anti-rabbit conjugate as described above.

The antisera used, with exception of the mouse monoclonals, were affinity-purified and obtained from MIAB, Uppsala, Sweden. All sera were run in duplicates and the intraassay variation for IgG, IgA, IgG1 and IgG4 were 7%, 8%, 9%, and 8% respectively. p-Nitrophenyle-phosphate was used as the substrate, and extinction at 405 nm was read with a KONTRON spectrophotometer. A reference curve was run in parallel on each plate using a reference serum. All data were expressed in relation to this serum which was arbitrarily given the contents of 100 U antibody, irrespective of class and subclass assayed.

Timothy-specific IgG antibodies were also measured by a sandwich technique using Sepharose protein-A for adsorption of IgG from the serum samples (6). ^{125}I-labelled allergen was added and after washing the bound radioactivity was measured in a gamma scintillation counter. The uptake was expressed in parts/1000 of that of a reference serum. In this assay a partially purified timothy pollen preparation obtained from Pharmacia AB, Uppsala, Sweden (Batch 780404) was used.

RESULTS

The timothy-specific IgE antibody concentrations are shown in Fig. 1. Evident variations are seen with high levels after the grass pollen seasons. Later in the years the IgE antibody levels subside. The interindividual spread in timothy-specific IgE level is about 30-fold and the individuals tend to remain at a similar level of IgE antibody concentration over the years.

The ELISA results of timothy-specific IgG (Fig. 2) showed clearcut seasonal variations which were not as pronounced as for IgE antibodies. These variations were more prominent in the patients with high levels of timothy-specific IgE antibody compared to those with low levels, in which antibody variations could be lacking entirely. The seasonal boosting effect could be registered as seasonal variations of both timothy-specific IgG1 and IgG4 (Figs. 3

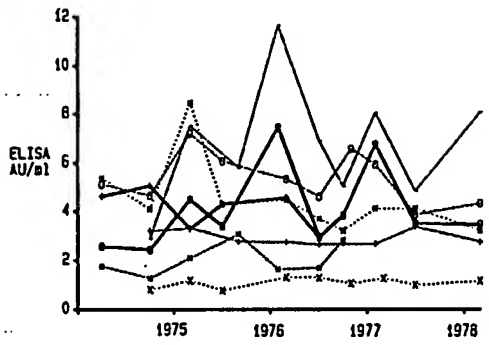

Fig. 2. Timothy-specific serum IgG antibody concentrations as measured by ELISA technique in eight atopic children during a 4-year period in relation to the pollen seasons. (Symbols see Fig. 1).

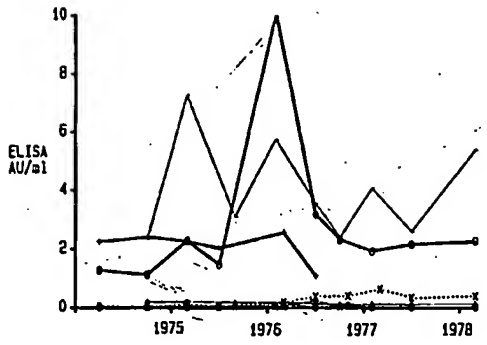

Fig. 4. Timothy-specific serum IgG4 antibody concentrations as measured with ELISA technique in eight atopic children during a 4-year period in relation to the pollen seasons. (Symbols see Fig. 1).

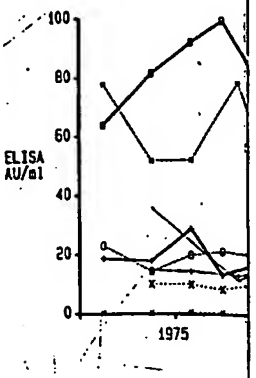

Fig. 6. Timothy-specific serum
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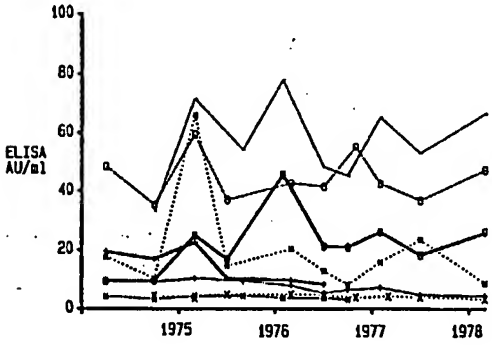

Fig. 3. Timothy-specific serum IgG1 antibody concentrations as measured with ELISA technique in eight atopic children during a 4-year period in relation to the pollen seasons. (Symbols see Fig. 1).

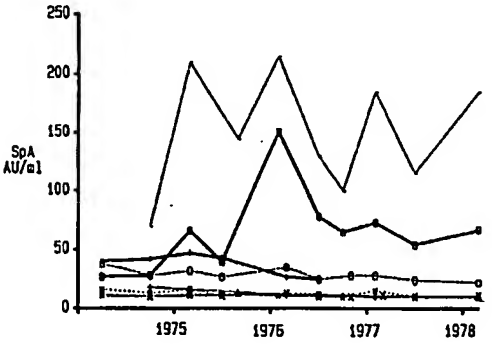

Fig. 5. Timothy-specific serum IgG antibody concentrations as measured with Sepharose protein-A technique in eight atopic children during a 4-year period in relation to the pollen seasons. (Symbols see Fig. 1).

and 4). Since the levels of IgG1 and IgG4 were both assigned arbitrary values without mutual correspondence, they can not be accurately compared. However, these variations in antibody levels were of a similar magnitude.

The results of the Sepharose protein-A assay of IgG antibody showed seasonal variations of a similar degree (Fig. 5). These were, however, less obvious in some patients than those obtained with the ELISA technique.

The corresponding contents of timothy-specific serum IgA antibody showed considerable interindividual variations. Seasonal boosting effects were, however, not registered as consistently as in the case of timothy-specific IgG antibody (Fig. 6).

DISCUSSION

Atopic sensitization is characterized by the occurrence of IgE antibody synthesis. It is a typical feature that exposure in the respiratory mucosal membranes of only minute amounts of antigen, e.g. of pollen origin, is sufficient for atopic sensitization in the predisposed (11). This sensitization, however, involves not only

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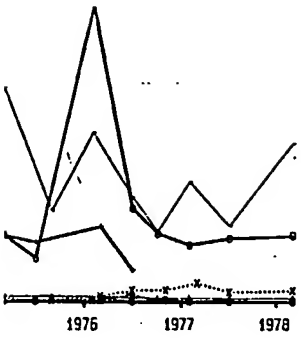

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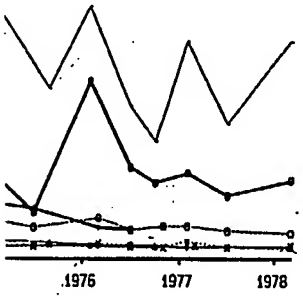

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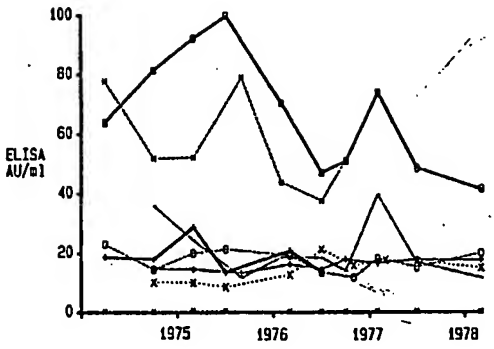

Fig. 6. Timothy-specific serum IgA antibody concentrations as measured with ELISA technique in eight atopic children during a 4-year period in relation to the pollen seasons. (Symbols see Fig. 1).

the synthesis of IgE antibody but also IgG and IgA antibody (15). The yearly seasonal pollen exposure causes the well-known variations of, e.g. the grass pollen specific IgE which is boosted postseasonally. The here presented results clearly show that timothy-specific IgG antibodies co-vary with IgE antibody of the same specificity and with the seasonal pollen exposure. This was true for IgG1 as well as IgG4 antibody which appeared to be boosted to a similar extent. Serum IgA antibody levels were not clearly boosted by the seasonal pollen load, even though there was some tendency.

Children are known to be more disposed to IgE response than adults, which is reflected in the peak level of total IgE in the early teen-ages (9). The children investigated here were of approximately this age group. Most probably, this age-related influence on the antibody response of respiratory mucosal antigen administration applies just as much for non-IgE as for IgE antibody. Concomitant decrease of mite and grass pollen-specific IgG and IgE antibody with age (16) favours this notion. It may thus be, that boostable IgG antibody responses by seasonal pollen exposure are more prominent in the young atopics. Pollen allergens are complex and timothy pollen is known to be composed of some 30 antigens, one third of which are known as allergens (17). The strongest IgE antibody responses are directed to two of these antigens, which are considered as major allergens (17). IgG antibodies are evoked primarily to those two substances in children given immunotherapy with timothy pollen allergen extracts (14), and most probably the naturally occurring IgG antibody responses are directed primarily to these two antigens.

The present data, showing a covariation of IgG and IgE antibody levels caused by seasonal pollen exposure, fit with other data showing correlating IgG and IgE antibody levels in individual sera (3), concomitant fall of IgE and IgG antibody to allergens during aging (16), and the finding of IgG and IgE antibody responses to the same components of complex allergens (12, 13, 14). These results together show that IgE antibody responses in the atopic are an integrated part of a complex immune response to allergens.

ACKNOWLEDGEMENTS

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Increased Ne in Exercise- a

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Exercise and inhalation bulized distilled water potent bronchoconstricto the characteristic bronch of asthmatic subjects.

The mechanism of ac unknown. Exercise-indu been related to water lo conditioning a change in lining the airways (3, 12 release of hypersensiti trigger a parasympath UNDW-induced bronch related to vagal reflex release (27, 29). Intere UNDW are inhibited b are associated with an i